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VIEWPOINT: PART OF A SPECIAL ISSUE ON PLANT CELL WALLS

Callose biosynthesis in arabidopsis with a focus on pathogen response: what we have learned within the last decade

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• **Background** (1,3)-β-Glucan callose is a cell wall polymer that is involved in several fundamental biological processes, ranging from plant development to the response to abiotic and biotic stresses. Despite its importance in maintaining plant integrity and plant defence, knowledge about the regulation of callose biosynthesis at its diverse sites of action within the plant is still limited. The moderately sized family of *GSL* (*GLUCAN SYNTHASE-LIKE*) genes is predicted to encode callose synthases with a specific biological function and subcellular localization. Phosphorylation and directed translocation of callose synthases seem to be key post-translational mechanisms of enzymatic regulation, whereas transcriptional control of *GSL* genes might only have a minor function in response to biotic or abiotic stresses.
• **Scope and Conclusions** Among the different sites of callose biosynthesis within the plant, particular attention has been focused on the formation of callose in response to pathogen attack. Here, callose is deposited between the plasma membrane and the cell wall to act as a physical barrier to stop or slow invading pathogens. Arabidopsis (*Arabidopsis thaliana*) is one of the best-studied models not only for general plant defence responses but also for the regulation of pathogen-induced callose biosynthesis. Callose synthase GSL5 (GLUCAN SYNTHASE–LIKE5) has been shown to be responsible for stress-induced callose deposition. Within the last decade of research into stress-induced callose, growing evidence has been found that the timing of callose deposition in the multilayered system of plant defence responses could be the key parameter for optimal effectiveness. This timing seems to be achieved through co-ordinated transport and formation of the callose synthase complex.

Key words: (1,3)-β-glucan, *Arabidopsis thaliana*, callose synthase, callose biosynthesis, plant cell wall polymer, innate immunity, microbial pathogens, papillae, pathogen response, plant defence, vesicle transport.

INTRODUCTION

Callose is a (1,3)-β-glucan cell wall polymer with some (1,6)-branches (Aspinall and Kessler, 1957). It is found in all multicellular green algae as well as higher plants (Scherp *et al.*, 2001). The amount and distribution of callose are highly variable depending on developmental stages and the presence of biotic as well as abiotic stresses.

During cytokinesis callose is transiently deposited in the cell plate of the phragmoblast. It has also been also associated with pollen self-incompatibility (Dumas and Knox, 1983) and pollen development (McCormick, 1993; Fei *et al.*, 2004). Callose is also an essential component of the transient cell wall surrounding pollen mother cells and encloses the four microspores after meiosis. In addition, callose forms a pre-cell wall at the growing pollen tube tip (Edlund *et al.*, 2004). Sieve plates, which are a basic component of the phloem, are already rich in callose under normal growing and developmental conditions (Hartig, 1851; Eschrich, 1956). When subjected to stress, callose accumulates rapidly and plugs the sieve pores. Similar to this stress response, callose biosynthesis and degradation in the neck region of plasmodesmata help to regulate permeability during abiotic and biotic stresses. In response to pathogen attack, callose is deposited between the plasma membrane and the pre-existing cell wall at sites of pathogen attack (Nishimura *et al.*, 2003).

Even though callose is involved in multiple, important biological processes in the plant, detailed knowledge about the regulation of this cell wall polymer and its specific function has not been provided for all diverse callose synthase family members. Major contributions to elucidate the biosynthesis and regulation of callose deposition were made by Hong *et al.* in 2001 and by Jacobs *et al.* and Nishimura *et al.* in 2003. They provided for the first time a detailed insight into the biological role of two callose synthase family members.

Apart from solving questions about stress-induced callose biosynthesis in general, the findings of Jacobs *et al.* (2003) and Nishimura *et al.* (2003) also raised new questions about the effectiveness and importance of pathogen-induced callose deposition at sites of infection. Because they showed enhanced pathogen resistance for arabidopsis mutants that were deficient in stress-induced callose deposition, callose was regarded as a possible by-product of the response to pathogen attack, without an important biological role in plant defence. Its ongoing utilization as marker for general alterations in pathogen defence responses or to screen new elicitors (McCann *et al.*, 2012) was not affected. This kind of usage benefited from the easy staining of this cell wall polymer with the fluorophore aniline blue

(Currier, 1957) in histological examination with an epifluorescence microscope with a UV filter, either with (Luna *et al.*, 2011) or without destaining of the plant tissue and in combination with fluorescent proteins or fluorescent dyes that are specific for distinct organelles or cellular structures (Xie *et al.*, 2012). Within recent years, several methods have been published that describe different approaches to the quantification of time-dependent callose formation to investigate the regulation of callose biosynthesis. They range from measuring callose intensity by counting white pixels of digital photographs or by calculating the number of depositions relative to the total number of pixels using office solutions like Photoshop (Luna *et al.*, 2011) or scientific software like ImageJ (Li *et al.*, 2009) to the application of automated analysis using the Acapella framework (Zhou *et al.*, 2012). The first successful application of super-resolution microscopy to aniline blue-stained callose deposits after fungal infection, which allows visualization of nanoscale, 3-D polymer networks (Eggert *et al.*, 2014), opens new possibilities in the histological examination of stress-induced structural modification of callose and its interaction with other cell wall polymers.

This article summarizes what is known about the regulation of callose synthase activity as well as what has been discussed with regard to this topic within the last decade based on results derived from new techniques and available mutant lines. We focus especially on the progress that has been made in understanding the regulation of callose biosynthesis in response to pathogen attack.

OVERVIEW OF THE ARABIDOPSIS CALLOSE SYNTHASE FAMILY

In most plants, the group of callose synthases encoding *GSL* (*GLUCAN SYNTHASE-LIKE*) genes forms a moderately sized gene family. The predicted function of *GSL*-encoded proteins as callose synthases is based on their homology with the yeast (*Saccharomyces cerevisiae*) *FKS* (*FK506 SENSITIVITY*) genes, which encode subunits of predicted (1,3)-β-glucan synthase complexes (Douglas *et al.*, 1994; Dijkgraaf *et al.*, 2002). In the best-studied model plant, arabidopsis (*Arabidopsis thaliana*), 12 *GSL* genes have been identified, which were initially designated as *GSL1*–*GSL12* (Richmond and Somerville, 2000). A parallel annotation referred to these genes as callose synthase genes *CalS1*–*CalS12* (Verma and Hong, 2001). The numerical designation has not been aligned in these two annotation approaches, which might result in confusion. Although a callose synthase function is very likely for most members of this gene family, direct biochemical evidence for callose synthase activity has not been provided yet, which prompted us to continue using the conservative *GSL* nomenclature. A comparative list of the parallel annotations is provided in Table 1 together with additional information on their individual biological roles (as far as known).

Subcellular localization of callose synthases

A minimum of ten transmembrane domains were predicted for all 12 members of the arabidopsis callose synthase family using the ARAMEMNON database (Schwacke *et al.*, 2003) and the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM), which would imply a membrane localization and has already been confirmed for eight callose synthases. Most experimental data have been derived from membrane preparations followed by mass spectrometry analysis (Table 1). In addition to a general localization in membranes, callose synthases might accumulate in detergent-resistant membrane fractions, the so-called lipid rafts, as recently shown for callose synthases from cultured poplar (*Populus trichocarpa*) cells (Srivastava *et al.*, 2013). However, accumulation of callose synthases in lipid rafts could be plant-specific, because unequal distribution could not be detected in arabidopsis using either cell-fractioning experiments or callose synthases tagged with fluorescent proteins. In this regard, the arabidopsis callose synthases GSL2, GSL5 and GSL6 were successfully fused with a fluorescent protein. The green fluorescent protein (GFP)-tagged GSL2 and GSL5 co-localized with FM4-64, a lipophilic fluorescent dye and plasma membrane marker (Bolte *et al.*, 2004), without showing preferences for putative membrane regions. In addition, GSL2 (Xie *et al.*, 2012) and GSL5 (Drakakaki *et al.*, 2012; Ellinger *et al.*, 2013) were also found in vesicle-like structures, which indicates a possible transport mechanism for callose synthases to sites of required callose biosynthesis, raising questions about putative regulatory pathways involved in targeted translocation.

Biological role of callose synthase family members in pollen fertility and plant development

A reason for the limited knowledge of the biological role of specific callose synthases can be found in the lack of phenotypes for specific *gsl* disruption mutants, which could indicate partially redundant functions. Especially in plant growth and development, experimental data support the assumption of possible redundancy. Callose synthases encoded by *GSL1*, *GSL2*, *GSL5*, *GSL8* and *GSL10* were required for callose biosynthesis during pollen development and were essential for pollen fertility and/or viability. GSL1 and GSL5 were required for the formation of the callosic cell wall that separates the microspore in the tetrad and for subsequent pollen grain maturation (Enns *et al.*, 2005). The degeneration of microspores in *gls2* disruption mutants indicated that this callose synthase would be required for exine formation during microgametogenesis (Dong *et al.*, 2005). The disruption mutants *gsl8* and *gsl10* showed perturbation in the symmetry of microspore division and had irregular callose deposition during microgametogenesis (Töller *et al.*, 2008). In addition, the *GSL8*- and *GSL6*-encoded callose synthases play a role in forming premature cell walls at cell plates of dividing cells. Together with GSL12, GSL8 predominantly contributes to callose deposition at plasmodesmata (Guseman *et al.*, 2010; Sevilem *et al.*, 2013). So far, only GSL7 has been shown to be responsible for the synthesis of callose in sieve plate pores (Barratt *et al.*, 2011; Xie *et al.*, 2011).

Biological role of callose synthase family members in stress and pathogen response

Regarding stress-induced callose biosynthesis, *GSL5* (in the context of pathogen response first described as *PMR4*; *POWDERY MILDEW RESISTANT4*) encodes the callose synthase that is responsible for the deposition of callose in papillae, which are cell wall thickenings at sites of pathogen attack and at

TABLE 1. *Overview of subcellular localization and biological role of callose synthases encoded by the* GSL *gene family in* Arabidopsis

GSL[1]	CalS[2]	Gene ID[3]	Subcellular localization (experimental)	Biological function
Biological role in fertility and cell division				
GSL1	CalS11	AT4G04970	MS/MS: plasma membrane (Benschop *et al*., 2007)	Pollen development and fertility (Enns *et al*., 2005)
GSL2	CalS5	AT2G13680	GFP-tagged protein in cultured tobacco BY-2 cells: plasma membrane and Golgi-related endo-membranes (Xie *et al*., 2012)	Found in mature pollen grains (Grobei *et al*., 2009); involved in late stages of pollen development and pollen tube (Dong *et al*., 2005; Xie *et al*., 2010)
GSL6	CalS1	AT1G05570	GFP-tagged protein: cytosol and plasma membrane (Hong *et al*., 2001*a*); MS/MS: plasma (Alexandersson *et al*., 2004; Keinath *et al*., 2010; Benschop *et al*., 2007; Zhang and Peck, 2011)	Required for callose depositions during cell plate formation (Hong *et al*., 2001*a*, *b*)
GSL8	CalS10	AT2G36850	MS/MS: plasma membrane (Alexandersson *et al*., 2004; Mitra *et al*., 2009; Benschop *et al*., 2007; Marmagne *et al*., 2007; Zhang and Peck, 2011)	Required for male gametophyte development and plant growth (Töller *et al*., 2008); entry of microspores into mitosis (Chen *et al*., 2009; De Storme *et al*., 2013); required for callose biosynthesis at the cell plate (Thiele *et al*., 2009), involved in stomatal pattering and deposition at the plasmodesmata (Guseman *et al*., 2010; Han *et al*., 2014)
GSL10	CalS9	AT3G07160	MS/MS: plasma membrane (Alexandersson *et al*., 2004; Dunkley *et al*., 2006; Benschop *et al*., 2007; Marmagne *et al*., 2007; Mitra *et al*., 2009; Keinath *et al*., 2010; Zhang and Peck, 2011)	Required for male gametophyte development and plant growth (Töller *et al*., 2008); together with GSL8, involved in entry of microspores into mitosis (De Storme *et al*., 2013)
Structural reinforcement				
GSL5 (PMR4)	CalS12	AT4G03550	GFP-tagged protein: plasma membrane (Drakakaki *et al*., 2012; Ellinger *et al*., 2013); MS/MS: plasma membrane (Alexandersson *et al*., 2004; Dunkley *et al*., 2006; Benschop *et al*., 2007; Mitra *et al*., 2009; Keinath *et al*., 2010; Zhang and Peck, 2011)	Required for wound and papillary callose formation in response to fungal pathogens (Jacobs *et al*., 2003; Nishimura *et al*., 2003; Ellinger *et al*., 2013; Naumann *et al*., 2013); important for exine formation and pollen wall patterning (Enns *et al*., 2005)
GSL7	CalS7	AT1G06490	No experimental data	Responsible for callose deposition in the phloem (Barratt *et al*., 2011; Xie *et al*., 2011)
GSL12	CalS3	AT5G13000	MS/MS: plasma membrane (Benschop *et al*., 2007; Keinath *et al*., 2010; Zhang and Peck, 2011)	Required for callose deposition at plasmodesmata (Sevilem *et al*., 2013)
Unknown function				
GSL3	CalS2	AT2G31960	MS/MS: plasma membrane (Alexandersson *et al*., 2004; Benschop *et al*., 2007; Kierszniowska *et al*., 2009)	Unknown function
GSL4	CalS8	AT3G14570	No experimental data	Unknown function, found in roots (Lan *et al*., 2011)
GSL9	CalS4	AT5G36870	No experimental data	Unknown function, found in leaf membranes (Mitra *et al*., 2007)
GSL11	CalS6	AT3G59100	No experimental data	Unknown function
GSL1	CalS2	Gene ID3	Subcellular localization (experimental)	Biological function

[1]Annotation according to Richmond and Somerville (2000).
[2]Annotation according to Verma and Hong (2001).
[3]Gene identifier according to The Arabidopsis Information Source (http://www.arabidopsis.org).

wounding sites (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003; Kim *et al.*, 2005). In addition to redundancies in callose biosynthesis in developmental processes, there is growing evidence that, apart from GSL5, at least one additional callose synthase could be involved in callose deposition after treatment with purified elicitors from callose-inducing pathogens. The treatment of arabidopsis leaves with chitosan, which is an elicitor associated with fungal pathogens (El Hadrami *et al.*, 2010), also resulted in callose deposition in *GSL5* disruption mutants. Comparison with elicitor-induced callose production in wild-type plants revealed that ~10 % of the callose produced was derived from callose synthase(s) other than GSL5. In contrast, callose deposition induced by flg22, an elicitor derived from the flagellin of bacterial pathogens (Gomez-Gomez *et al.*, 1999), was entirely dependent on GSL5 activity (Luna *et al.*, 2011). Although a redundant callose synthase for pathogen- or elicitor-induced callose formation has not been identified, induction of gene expression was observed for *GSL5* and also for *GSL6* and *GSL11* after biotic stress (Jacobs *et al.*, 2003).

Based on their biological roles, the GSL callose synthase family can be divided into two separate groups. The larger one, including GSL1, GSL2, GSL6, GSL8 and GSL10, is mainly involved in callose biosynthesis during pollen development and cell division. Members of the smaller group, including GSL5, GSL7 and GSL12, are required when callose acts in plugging, barrier formation or other kinds of structural reinforcement. The function of the remaining members, GSL3, GSL4, GSL9 and GSL11, is still unknown. Involvement in a precise biological process and their localization have not been determined yet.

REGULATION OF CALLOSE BIOSYNTHESIS

A common characteristic of the majority of the described callose synthases is their strict temporal and spatial regulation, which is required so that they can fulfil a specific biological function. This leads directly to the question of the regulatory mechanisms that control callose biosynthesis.

Regulation at transcriptional level

Overlapping expression patterns of several *GSL* genes were observed in response to wounding and physiological stresses as well as in different tissues during plant development (Dong *et al.*, 2008). However, in almost all of these cases of possible transcriptional regulation of *GSL* genes, alterations of gene expression were relatively moderate and did not exceed a 2·5-fold induction compared with controls, based on our analysis of publicly available expression data provided in the Genevestigator database (Hruz *et al.*, 2008). Exceptions to these moderate inductions were treatments with cycloheximide, which is an inhibitor of protein biosynthesis (Ellis and Macdonald, 1970), and salicylic acid, a phenolic compound that is important for the regulation of multiple physiological processes and plant defence (An and Mou, 2011). Cycloheximide induced up to 50-fold upregulation of *GSL3* expression and salicylic acid treatment induced strong *GSL5* and *GSL6* expression (Dong *et al.*, 2008) regulated by the salicylic acid receptor NPR1 (NON-EXPRESSOR OF PATHOGENISIS-RELATED GENES 1) (Wu *et al.*, 2012). A significant increase in *GSL6* expression was also observed after infection with different bacterial *Pseudomonas syringae* pathovars and the downy mildew *Hyaloperonospora arabidopsidis.* Based on these expression results, regulation of callose biosynthesis at the transcriptional level seems to be restricted to specific stress situations. However, a transcription factor for the regulation of *GSL* gene expression in the biotic or abiotic stress response is not known yet. An exception is auxin-induced, callose-mediated plasmodesmatal gating, in which *GSL8* expression is regulated by the auxin response factor ARF7 (Han *et al.*, 2014). Another auxin response factor, ARF17, was recently shown to regulate the expression of *GSL2* during pollen wall pattern formation (Yang *et al.*, 2013). In addition to ARF17, *GSL2* expression seemed to be regulated by pre-mRNA splicing through CYCLIN-DEPENDENT KINASE G1 (CDKG1) during pollen wall formation (Huang *et al.*, 2013). Because the expression of *GSL2* was down-regulated in both *cdkg1* and *arf17* disruption mutants, transcriptional regulation of callose biosynthesis seems to be important during pollen wall pattern formation.

Regulation by phosphorylation

A post-translational modification that has been discussed as a putative mechanism of regulating callose biosynthesis is phosphorylation. In yeast, the activity of the callose synthase homologues FKS1 and FKS2 was dependent on their phosphorylation status (Qadota *et al.*, 1996; Calonge *et al.*, 2003; Ishiguro *et al.*, 2013). Regulation through phosphorylation was also proposed for the arabidopsis callose synthases GSL10, where phosphorylated peptides were identified by mass spectrometry after treatment with elicitor flg22 and xylanase (Benschop *et al.*, 2007), and GSL12 (Nuhse *et al.*, 2003). A GSL5 peptide was found in six independent experimental approaches with phosphorylation at the same serine residue after various stress situations (Nuhse *et al.*, 2007; Sugiyama *et al.*, 2008; Reiland *et al.*, 2009, 2011; Kline *et al.*, 2010; Nakagami *et al.*, 2010). However, a kinase or phosphatase that would regulate the phosphorylation status of a callose synthase in response to stress or at a specific developmental stage has not been identified yet.

Regulation by complex formation

Regulating substrate uptake into the catalytic centre, either by conformational changes or substrate delivery, is another common mechanism of the regulation of enzyme activity. This type of regulation usually depends on accessory proteins interacting with the callose synthase. The formation of high molecular callose synthase complexes with accessory, putative regulatory proteins was first predicted from experiments with yeast (Qadota *et al.*, 1996) and green algae (Stone, 2006). In plants, the arabidopsis callose synthase GSL6 was partially purified with two cell plate-associated proteins, phragmoplastin and the UDP-glucose transferase UGT1 (Hong *et al.*, 2001*b*). UGT1 also interacted with Rop1, a Rho-like GTPase. Interestingly, this interaction occurred only in the GTP-bound configuration of Rop1, which suggests that the plant callose synthase might be regulated by Rop1 by interaction with UGT1 (Hong *et al.*, 2001*b*). A monomeric GTPase from the Rho family was also involved in callose biosynthesis in yeast (Calonge *et al.*, 2003). Finally, an annexin-like protein modulated callose synthase activity in cotton (*Gossypium hirsutum*) (Andrawis *et al.*, 1993). In summary, the hypothetical callose synthase complex proposed by Verma and Hong in 2001, in which the hydrophilic loop may interact with a monomeric GTPase, UGT, annexin and a sucrose synthase, is still a widely accepted model.

Regulation by transport

Phosphorylation and interaction with other proteins might also be involved in the transport and focal accumulation of callose synthases at the various sites of callose biosynthesis. It is known that these regulatory processes are required for the correct timing and amount of callose deposition. Verma and Hong (2001) proposed that Rho-like GTPase might not only regulate callose synthase activity but also function as a spatial regulator. Another well-documented example of a transport process is the production of callose at the growing tip of pollen tubes. Transport of callose synthases in tobacco pollen tubes seemed to start at the endoplasmic reticulum, where the enzyme might be synthesized or processed. Subsequently, they were proposed to be integrated into Golgi bodies and transported along bundles of actin filaments to the subapex of the pollen tube (Cai *et al.*, 2011). These finding were based on the inhibition of vesicle transport. Most knowledge about the transport of callose synthases and the underlying regulatory mechanisms applies to callose biosynthesis at the tips of growing pollen tubes and the stress-induced callose synthase GSL5 from arabidopsis, for which the current discussion about the regulation of plant defence responses is summarized in the following section.

CURRENT VIEWS REGARDING INDUCTION AND REGULATION OF GSL5 IN PLANT–PATHOGEN INTERACTION

Callose biosynthesis in response to plant–bacteria interaction

Apart from abiotic stress and wounding (Wheeler, 1974; Ryals *et al.*, 1996; Jacobs *et al.*, 2003; Mauch-Mani and Mauch, 2005), a wide range of bacteria induce callose deposition in leaf epidermal cells. Based on studies with bacterial elicitors, several pathways were identified that induced callose biosynthesis

and depended either on the production of reactive oxygen species (Luna *et al.*, 2011) and salicylic acid (Nishimura *et al.*, 2003; Flors *et al.*, 2008) or the accumulation of indole glucosinolates (Geng *et al.*, 2012). However, precise analysis of the role of GSL5 in basal resistance or innate immunity to bacterial pathogens is generally restricted by the fact that *GSL5* disruption mutants revealed a hyperinduction of salicylic acid biosynthesis as well as constitutive expression of plant defence-related genes (Nishimura *et al.*, 2003). In addition, neither the lack of callose deposition nor enhanced callose deposition alone was sufficient to increase resistance to bacterial pathogens (Moreau *et al.*, 2012). Therefore, the biological role of callose in plant–bacteria interaction is still a controversial issue in current discussions. Besides functioning as a physical barrier to prevent ingress of pathogens (Ellinger *et al.*, 2013), callose might form a diffusion barrier (Aist, 1976; Samardakiewicz *et al.*, 2012) or could be involved in the detoxification of antimicrobial compounds (Luna *et al.*, 2011).

Although accumulation of callose in response to pathogen-associated molecular pattern (PAMP) recognition might not primarily contribute to pathogen resistance, this plant defence response can be used to study the regulation and transport of callose synthases. The fast response of a plant to pathogen attack relies on its innate immunity (Jones and Dangl, 2006), which can be divided into two arms: (1) PAMP-triggered immunity (PTI) (Boller and Felix, 2009); and (2) effector-triggered immunity (Senthil-Kumar and Mysore, 2013). PAMPs – or, more generally, MAMPs (microbial associated molecular patterns) – are highly conserved molecular elicitors derived from microbial pathogens. The most prominent bacterial MAMPs, flg22 (Gomez-Gomez *et al.*, 1999; Luna *et al.*, 2011), the elongation factor Tu (EF-Tu) (Lu *et al.*, 2009), lipopolysaccharides (Keshavarzi *et al.*, 2004; Sun *et al.*, 2012) and peptidoglycan hairpins (Erbs *et al.*, 2008; Erbs and Newman, 2012), all elicit callose deposition. MAMPs are recognized by a class of specific plasma-membrane-bound extracellular receptors, the so-called pattern recognition receptors (PRRs) (Dodds and Rathjen, 2010; Beck *et al.*, 2012). MAMP-induced activation of PRRs triggers a series of fast defence responses, which include the production of reactive oxygen species, the induction of mitogen-activated protein kinases (MAPKs) and changes in protein phosphorylation, and were detectable already within the first 5 min after PRR activation. This first wave of responses is followed by ethylene and glucosinolate biosynthesis (Clay *et al.*, 2009), receptor endocytosis (Beck *et al.*, 2012) and induction of gene expression. Callose deposition at infection sites is normally observed within hours after pathogen attack and is therefore classified as a late PTI response. Interestingly, we observed not only callose deposition related to a late PTI response starting 6 h after infiltration of 1 μM flg22 into adult arabidopsis leaves, but also a relatively fast callose response 60–90 min after flg22 treatment (Fig. 1). This early callose response to flg22 treatment is commonly not recorded because in most studies visualization of callose started 18–24 h after treatment. However, also in studies with an early start of flg22-induced callose detection, such as that of Luna *et al.* (2011), an early callose response was not detected. Differences in the occurrence of an flg22-induced, early callose response could be determined by the method of treatment and the physiological and developmental stage of the plant. Whereas we infiltrated 4-week-old arabidopsis leaves, Luna *et al.* (2011) added a 1 μM flg22 solution to the growth medium of 9-day-old arabidopsis seedlings.

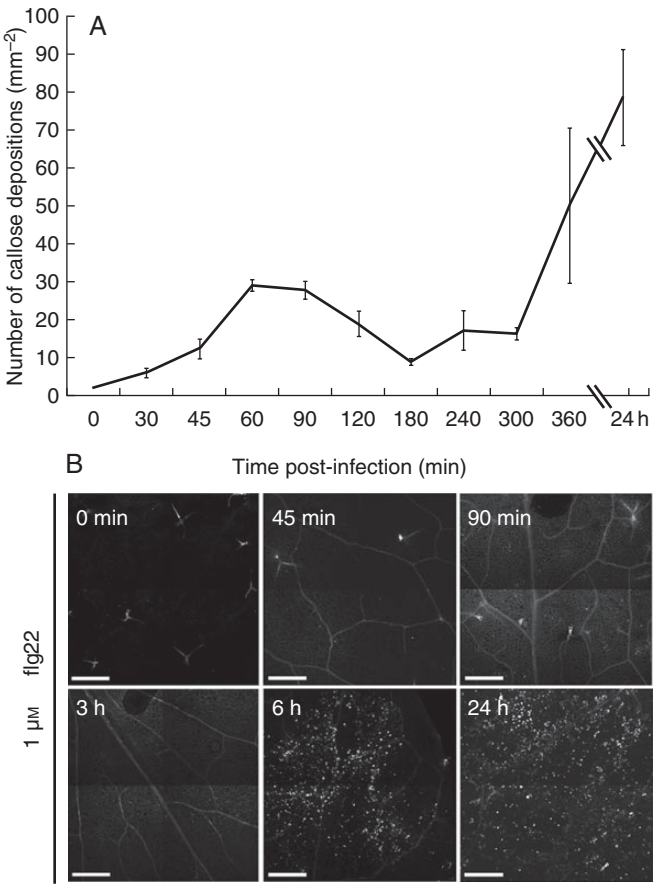

FIG. 1. Callose deposition in response to flg22 infiltration. For each time-point, three 4-week-old arabidopsis leaves were infiltrated with 1 μM flg22 solution as described in Daudi *et al.* (2012) and harvested at the indicated time-points after treatment. Chlorophylls were removed with ethanol to eliminate the autofluorescence background in callose visualization with the organic fluorophore aniline blue (Stein *et al.*, 2006). Micrographs were taken by confocal laser-scanning microscopy using a 405 nm diode laser for aniline blue excitation. Emission filtering was achieved using a 472- to 490-nm bandpass filter. (A) Number of callose depositions per mm^2 counted by CalloseMeasurer (Zhou *et al.*, 2012). Data are means of three independent experiments with $n = 4$. Error bars indicate standard deviation. (B) Distribution and amount of callose depositions in leaves stained with aniline blue at indicated time-points after flg22 infiltration. Scale bars = 500 μm.

We previously observed the ability for fast callose deposition in response to stress also in arabidopsis lines with *GSL5* overexpression. Epidermal leaf cells of the overexpression line strongly deposited callose 60 min after spraying flg22, in contrast to wild-type and *pmr4* lines without an early callose response (Ellinger *et al.*, 2013). These results clearly indicate that initial callose biosynthesis is GSL5-dependent, can be explained by the presence of this enzyme at plasma membrane before treatment, and occurs without *de novo* protein biosynthesis. We hypothesize that callose deposits observed 6 h after flg22 treatment and later might be mainly derived from transported GSL5 because we did not detect induction of *GSL5* expression at this time-point (data not shown). In this regard, Wang and Forbert (2013) also did not find a correlation between callose deposition and *GSL5* expression after flg22 infiltration of arabidopsis leaves.

Reduction in callose deposition between 120 and 300 min after flg22 spraying may be due to degradation of callose, which we also observed after fungal infections at the first callose deposition (Ellinger *et al.*, 2013).

In addition to studying callose biosynthesis during PTI, analysis of this plant defence response during effector-triggered immunity can provide new insight into the regulation of this process during pathogen attack. As mentioned before, PTI results in callose deposition at the cell wall, but microbial effectors targeting PTI can suppress callose deposition (Hauck *et al.*, 2003; Underwood *et al.*, 2007; Zhang *et al.*, 2007; Fabro *et al.*, 2011). Biotrophic bacterial pathogens have evolved and maintained a type III secretion system to deliver effectors into host cells to suppress elicitor-induced defence responses (Lee *et al.*, 2013). The pathogenic bacterium *P. syringae* pv. *tomato* DC3000 secretes the effector proteins Hrp outer protein M1 (HopM1) and coronatine, which structurally mimics active jasmonic acid conjugates. Both effectors target and inhibit distinct signalling steps to suppress callose deposition, which is independent of salicylic acid responses but dependent on the accumulation of indole glucosinolates (Geng *et al.*, 2012). In addition, HopM1 suppresses PTI responses by interfering with vesicle trafficking (Nomura *et al.*, 2011). Pathogen-induced degradation of the trans-Golgi network seems to be critical for invading bacteria to overcome the plant's effector-triggered immunity mechanism for successful colonization. The trans-Golgi network and early endosomes function as a central junction for major endomembrane trafficking events, which are required not only for endocytosis but also for the secretion of apoplastic proteins such as the pathogenesis-related protein PR1 (Wang *et al.*, 2005; Gu and Innes, 2012). Because bacteria-induced callose accumulation was delayed in arabidopsis mutants that were impaired in vesicle-associated secretion processes (Kwon *et al.*, 2008) and their regulation at transcriptional level (Wang and Fobert, 2013), these regulatory mechanisms might also apply to the transport of GSL5, as observed in plant–fungus interaction (Nielsen *et al.*, 2012).

Callose biosynthesis in response to plant–fungus interaction

A further example of highly localized callose accumulation is the deposition of callose in papillae in response to fungal attack at sites of attempted penetration in epidermal cells (Zimmerli *et al.*, 2004; Koh *et al.*, 2005; Nielsen *et al.*, 2012; Ellinger *et al.*, 2013). After powdery mildew infection of arabidopsis leaves, the pathogen-induced callose synthase GSL5 was shown to be recruited from the plasma membrane, where it localized in untreated leaf epidermal cells, to the site of attempted fungal penetration. Here, it was reintegrated into the plasma membrane to generate localized callose plugs (Ellinger *et al.*, 2013). A general transport of callose synthases in the vesicles is also supported by a study by Drakakaki *et al.* (2012), in which biochemical analysis revealed the presence of GSL5 in the SYP61 trans-Golgi network compartment.

Involvement of transport processes in callose accumulation during papilla formation after fungal attack was also observed by Nielsen *et al.* (2012). Treatment of leaves with brefeldin A, which is a fungal inhibitor of vesicle transport (Sciaky *et al.*, 1997), inhibited callose accumulation in the papilla. They further proposed that papilla formation would require rapid reorganization of material from the plasma membrane, which might be sorted into multi-vesicular bodies and directed to the site of fungal attack (Nielsen *et al.*, 2012).

Exocytosis mediated by multi-vesicular bodies is well studied in animals (Harding *et al.*, 1983; Pan and Johnstone, 1983). In plants, a retrograde pathway has been proposed that would involve a fusion of multi-vesicular bodies with the plasma membrane, resulting in the delivery of previously intralumenal vesicles to the cell exterior. In barley as well as in arabidopsis, putative multi-vesicular bodies were observed in close contact with the plasma membrane in TEM experiments (An *et al.*, 2006; Micali *et al.*, 2011). However, callose was not detectable in multi-vesicular bodies and vesicles in the central vacuole during microscopy of fixed tissue (An *et al.*, 2006). Further evidence that multi-vesicular bodies play a role in the delivery and assembly of callose and other defence components at the growing papilla was derived from studies with barley in response to the powdery mildew *Blumeria graminis* f. sp. *hordei* (Böhlenius *et al.*, 2010). In this pathosystem, a monomeric G-protein from the ADP ribosylation factor (ARF) GTPase (ARFA1b/1c) was associated with plant multi-vesicular bodies and was required for penetration resistance and callose deposition. However, ARFA1b/1c was not required to form the basic structure of papillae. Although ARFA1b/1c mainly localized to Golgi and endocytotic vesicles, it was also identified as a component of multi-vesicular bodies, which might indicate involvement in vesicle budding and callose deposition in papillae. Additional evidence for this hypothesis derived from mutant lines of the ADP ribosylation factor–GTP exchange factor (ARF-GEF) GNOM. The analysis of a partially functional mutant revealed a delay in papilla formation and callose accumulation as well as reduced penetration resistance. Moreover, a time-course study revealed that these *gnom* mutants had a delay of ~30 min in the appearance of callose (Nielsen *et al.*, 2012). Combining these results with recent studies of callosic papillae at the site of fungal penetration using super-resolution microscopy (Eggert *et al.*, 2014), it becomes evident that precise timing of pathogen-induced callose biosynthesis is one of the key factors in callose-mediated resistance against biotrophic fungi. The complete penetration resistance of GSL5-overexpressing lines to powdery mildews is based on elevated and massive physical strengthening of the cell wall at infection sites (Ellinger *et al.*, 2013, Naumann *et al.*, 2013), which includes the formation of a physical barrier against pathogen-secreted cell wall hydrolases (Eggert *et al.*, 2014).

CONCLUSIONS AND FUTURE PROSPECTS

Direct evidence for a specific regulatory mechanism of callose biosynthesis in plants has only been provided for pollen wall pattern formation, in which transcriptional regulation of callose formation is very likely. There is also growing evidence for the requirement of transport processes in pathogen-induced callose biosynthesis. Figure 2 summarizes all discussed facts about callose biosynthesis and its regulation in four main spheres of action, which are surrounded by factors that might influence callose regulation related to the specific action, but have not been determined yet. Figure 2 illustrates that profound knowledge already exists, but similar questions are still open within the different spheres of callose action. Also, much basic

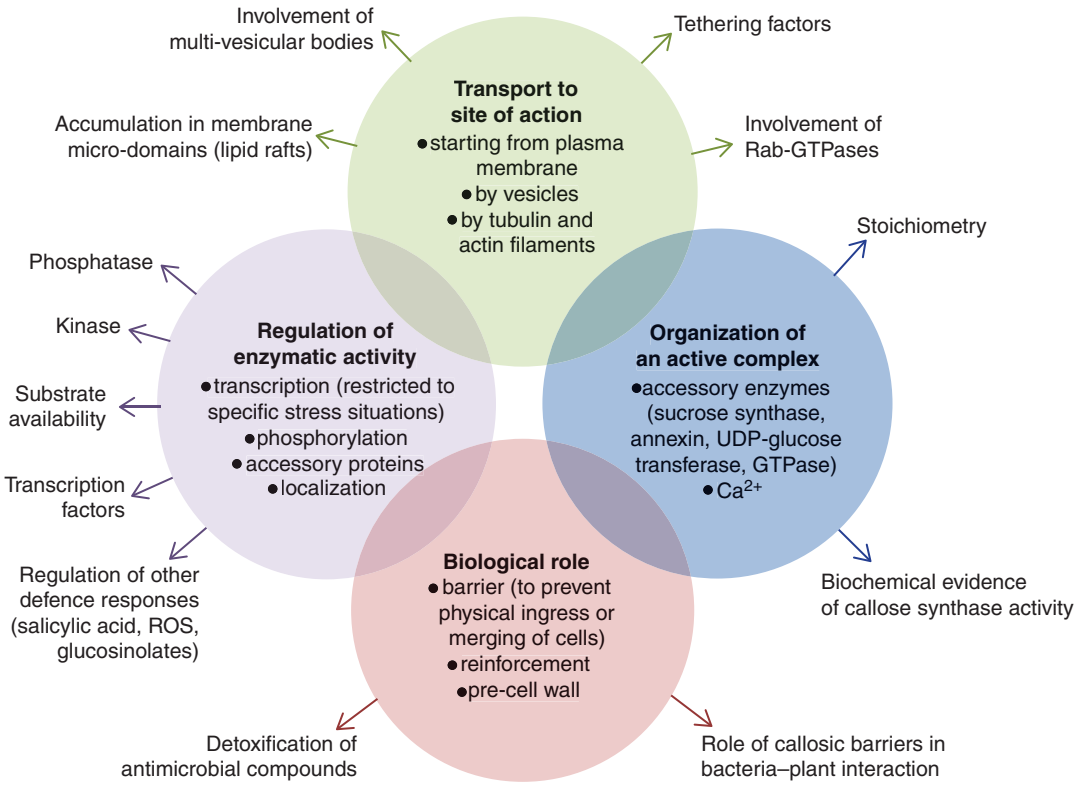

FIG. 2. Spheres of action in callose biosynthesis and regulation. Overview of four major spheres of action involved in callose biosynthesis and its regulation. Inside the spheres: aspects described for callose biosynthesis are described inside the spheres; aspects requiring further work in future projects (discussed within the text) are described outside the spheres.

information, such as direct biochemical proof of the callose synthase activity of all 12 GSL members and the stoichiometry of the active callose synthase complex, is still missing. From our point of view, a milestone in testing and understanding the regulation of callose biosynthesis would be the purification of an active complex or subunit from the plasma membrane or its heterologous expression and subsequent *in vitro* analysis. Nevertheless, promising strategies have already been established that will promote research on callose biosynthesis, and include the application of super-resolution microscopy (Eggert *et al.*, 2014) and the use of cultured cells (Srivastava *et al.*, 2013).

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